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Article

Analytical method for the determination of Hg from the organs of some terrestrial plants using cold vapor atomic absorption spectrometry

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Abstract
The aim of the study was to develop a new method for mercury determination from plants grown on mercury-polluted soils as well as to evaluate the mobility and bioavailability of mercury in the soil and its transfer to different plant organs (root, stem, leaves) from three different plants, such as mustard (Sinapis alba), thyme (Thymus serpyllum) and wild thyme. A cold vapor atomic absorption spectrometry (CV-AAS) method for detection of mercury in plants was developed. The developed method was validated in the laboratory and was verified using two different certified reference materials (CRMs) type matrix, namely: NIST 1515 Apple Leaves and BCR-482 Lichen.

Keywords: *mercury, CVAAS, plants, organic mercury, inorganic mercury*

INTRODUCTION

Soil pollution with mercury represents a serious environmental pollution problem worldwide. Unlike most organic contaminants that lose their toxicity with biodegradation, mercury can lead to long-term toxic effects, because it is not degraded and can be transferred to plants, thus affecting the health of humans and animals [1-3]. Mercury and its compounds are among the most toxic pollutants in the environment, having carcinogenic, teratogenic, neurotoxic and bioaccumulative effects that cause damage to both the human body and the ecosystem. As a result of these toxic effects, mercury pollution poses a risk to human health through its potential to contaminate the food chain (plant → animal → human being). According to ASTDR, 2015 (Agency for Toxic Substances and Disease Registry), mercury ranks 3rd in the list of priority dangerous substances.

Because it is used in numerous industrial processes, mercury is released in large quantities in different environmental compartments, such as soil, surface water, wastes, atmospheric particles [4-10]. In addition, due to the chloroalkane industry, significant mercury pollution of water, air and fish was found in Russia (Chlor-alkali plant JSC "Kaustic" Volgograd, Chlor-alkali plant Usoliekhimprom), Czech Republic (Chlor-alkali plant Spolana Neratovice) [11]. Besides the chloroalkane industry, the areas that pollute the most with mercury are mining areas (Nikitovsky Mercury mine plant, Gorlovka, Ukraine, Khaidarkan mercury mine, Kyrgyzstan, Abandoned mercury lamp plant, Rio de Janeiro, Brazil, LSM, Australia [12].

Phytoremediation is a technology for the elimination of environmental pollutants that can be applied to both organic pollutants (pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons) and inorganic pollutants (especially heavy metals, radioactive isotopes) present in soil, water or air [13]. Therefore, the term phytoremediation refers to a diverse range of plant-based technologies, which use either natural plants or genetically modified plants, to remediate contaminated environments.

The phytoremediation process uses green species (*Thlaspi caerulescens, Brassica juncea, Helianthus annuus*) and woody species (*Salix spp, Populus spp*) because they can remove, absorb various environmental contaminants, among those listed above, from soil or water, due to their capacity to transport and accumulate contaminants. In addition, phytoremediation makes it possible to avoid the excavation of contaminated sites, reduces the risk of contaminant dispersion and is applicable for the decontamination of sites with several categories of pollutants [14, 15].

Hg, very toxic to plants, usually exists in mercuric (Hg^{2+}), elemental mercury (Hg^0) or alkylated (methyl/ethyl mercury) forms. The stability of Hg depends on pH and redox conditions. In oxidized conditions, mercury in mercuric forms is much more stable, while organic and/or inorganic Hg can be reduced to elemental form in mild reducing conditions. Then, it can be converted by biotic or abiotic processes into the most toxic forms, the alkylated ones. The most important mechanism for removing Hg from solutions is the adsorption process on sediments, soils and humus materials [16].

The toxicity of Hg in soil depends very much on its chemical species (organic/inorganic Hg species). In the environment, mercury exists in different forms such as: metallic mercury (Hg^0), inorganic mercury (HgS, $HgCl_2$) and organic mercury, especially methylmercury (MeHg). Methylation of mercury occurs, especially in anaerobic conditions [17]. The mercury present in the environment produces toxic effects, especially due to the conversion of inorganic mercury into organic mercury (especially methylmercury: MeHg), which is the most toxic form of mercury [18].

The speciation of mercury in the soil depends on the physical-chemical properties of the soil (pH, organic substances, extractive capacity of cations, etc.). The pH affects not only the bioavailability of mercury but also the dissolution of mercury by changing the composition of organic matter. Plants easily absorb mercury, especially in the root, because Hg is very soluble in water and easily transformed into the gas phase. Methylmercury is the form that has the greatest potential for bioaccumulation, being able to pass very easily through biological membranes and accumulate in cells.

The accumulation of mercury in the soil depends a lot on the properties of the soil, as well as on the type of cultivated plant and the speciation of mercury in the soil. Inside the plant, mercury can be found in different species and can be transported in different plant organs, but, in general, a predominant accumulation of this element was found in the root compared to the other plant organs. Mercury has toxic effects on plants even at low concentrations causing plant growth deficiency, inhibition of photosynthesis, generation of reactive oxygen species, damage to proteins and DNA. Currently, risk assessment and food safety have gained a lot of attention worldwide. Therefore, in addition to the toxic effects in plants, it is crucial to study the soil-plant-human transfer and the possible health risks associated with the consumption of food contaminated with mercury [19].

Although Hg has no physiological function in plants, it can exist in plants in both organic and inorganic forms, the former being much more toxic than the latter. It is known that plants, especially bryophytes and avascular plants are mercury accumulators, being able to release it back into the environment through the aerial parts of the plants [20].

Contamination of the environment with Hg can lead to contamination of the food chain, especially due to its accumulation in the edible plant parts of plants. Since mercury has no identified biological role and has genotoxic and carcinogenic potential, it is essential to monitor its biogeochemical behavior in the soil-plant system.

The aim of the study was to present a methodology for the determination of mercury in plants, later used in studies of Hg bioaccumulation in different medicinal plants. Common medicinal plants were selected and intoxicated with Hg in a laboratory controlled study.

EXPERIMENTAL PART

Materials

The seeds used were selected mustard (*Sinapis alba*) seeds (SIA 2302/18), selected thyme (*Thymus serpyllum*) seeds (AGROSEL, Lot no. 212105). In addition, the wild thyme seeds selected by the gardener (AGROSEL, Lot no. 313103) were purchased. All the selected seeds were seed that germinated and developed quality seedlings.

Ultra-trace nitric acid (69%, Supelco Merck, Darmstadt Germany), hydrochloric acid (30%, VWR Chemicals, Prolabo Canada), hydrogen peroxide (30%, Honeywell, Fluka, Germany) were used for treatment of the seeds and different organs of the plant. The chemical reagents used were of ultrapure quality, specific for the analysis of trace metals.
A universal soil amendment was used, a mixture of peat from medium to intense decomposed bogs, wood fibers, green compost, humus from tree bark, NPK fertilizer, sand. The universal soil, according to the information provided by the manufacturer, contains nitrogen (50-400 mg/L); P_2O_5 (50-200 mg/L); K_2O (50-200 mg/L); pH (5 – 7.5); organic substances (minimum 67%); humidity (60%); KCl less than 3 g/L.
Two sampled soils, one from the agricultural area adjacent to a pyrite waste storage dump (VC), respectively an agricultural soil located approximately 10 km from the pyrite waste storage dump (S) were collected from the depth of 0-20 cm.
Both the soils and the amendment were shredded, the woody parts, the remains of vegetation, the stones were removed. Then, the solid materials were sieved through a sieve with a mesh size smaller than 5 mm, to homogenize the entire quantity.
The purchased amendment was mixed with soil S from Calugareasca Valley agricultural land in a ratio of 2:3. In one large plastic container, were mixed in a ratio of 40% amendment and 60% soil from the Calugareasca Valley area (S). The resulted soil (M) was used in the translocation tests of mustard, thyme and wild thyme (control tests and enriched soil for mustard experiments). To determine the amount of soil, respectively of amendment, dry matter of each component was taken into account. The amendment had an average of 60%, dry matter, while the Calugareasca Valley soil had approximately 90% dry matter. The soil VC was divided in two parts (VC1, VC2) in order to a better homogenize of the soil mass.
For the Hg tests, certified reference material of 1000 mg/L Hg was used (CPAChem, Stara Zagora, Bulgaria).
All the solutions for standards and reagents were prepared using ultrapure water provided by Millipore Simplicity UV (Merck, Darmstadt, Germany) equipment.
Hg determination was performed with an ATI UNICAM 929 Atomic Absorption Spectrometer (Cambridge, UK) and an Atomic Fluorescence Spectrometer Quick Trace Mercury Analyzer M-8000 (Teledyne Cetac Technologies, Nebraska, USA).
Microwave oven ETHOS UP from Milestone, Italy was used for the pre-treatment step of soils and plant's organs.
Quality control of the results was performed with two different Certified Reference Materials (CRMs) type matrices, namely BCR-482 Lichen and NIST 1515-Apple leaves.

Experimental design
The first seedlings were planted in the middle of May 2021, two mustard seeds in each alveolus filled with amendment. For thyme and wild thyme were planted more seeds, because these are very small. The seedlings were watered with tap water without chlorine. The mini-greenhouses were covered and placed in a room with a constant temperature (28ºC). After a week, 48 mustard seeds germinated, being sufficient for the proposed experiments. For each thyme species, around 40 seedlings were obtained. The pictures of the seedlings are presented in figure 1.
In the mustard experiments was used soil M, which was enriched with two different concentration of Hg: 1mg/kg and 4 mg/kg. The Hg solutions were calculated taking into account the soil quantity and moisture, the initial concentration of the Hg in mixture soil and final concentration to be achieved. To enrich the soil S, calculated solutions of Hg were sprayed over a thin layer of soil, then and after adding another layer of soil Hg solution was sprayed again until and the solutions were finished and entire soil mass was sprayed. The soil was left to stabilize for 10 days, then mercury concentrations were determined and finally the enriched soils were used for seedlings.

a) b) c)

Fig. 1. Seedlings of: a) mustard, b) thyme and c) wild thyme

For each experiment, 2-3 blank tests were prepared, soils without added mercury, namely M1m, M2m, M1t, M2t, M1wt, M2wt. The tests for mustard (*Sinapis alba*) were performed at two different concentration: 1mg/kg Hg (T1), respectively 4 mg/kg Hg (T2) using S1 soil (mixture of amendment and Calugareasca Valley soil in a ratio of 2:3). The experiments were T1/1m and T1/2m for 1 mg/kg Hg, and T2/1m, T2/2m for 4 mg/Hg. In addition, for VC experiments, was used a soil from the polluted area so that the VC1m and VC2m experiment were carried out in an industrially contaminated soil with mercury.

Table 1. The mustard experiments

Experiments	Number of tests	Number of plants /tests	Pot type / soil mass	Pictures of the mustard experiments
Blank test M1m M2m	2	4	pot 18 cm^3, 1 kg	
T1/1m T1/2m	2	4	pot 18 cm^3, 1 kg	
T2/1m T2/2m	2	4	pot 18 cm^3, 1 kg	
VC VC1m VC2m	2	8	pot 30 cm^3, 5 kg	

Table 2. Thyme experiments

Experiments	Number of tests	Number of plants /tests	Pot type / soil mass	Pictures of the thyme experiments
Blank test M1t M2t	2	15-20	pot 30 cm^3, 5 kg	
VC VC1t VC2t	2	15-20	pot 30 cm^3, 5 kg	

Same soils were used also for thyme and wild thyme experiments, resulting VC1t, VC2t experiments, respectively VC1wt and VC2wt experiments for wild thyme. The number of tests, pot types, soil mass, number of plants for each experiment are presented in table 1 (mustard), table 2 (thyme) and table 3 (wild thyme).

Table 3. Wild thyme experiments

Experiments	Number of tests	Number of plants /tests	Pot type / soil mass	Pictures of the wild thyme experiments
Blank test M1wt M2wt	2	15-20	pot 18 cm^3, 1 kg	
VC VC1wt VC2wt	2	15-20	pot 30 cm^3, 5 kg	

Pre-treatment of soils and plant's organs

Great attention was paid to the samples (both soils and plants) during sampling, storage, and handling. The soil and plant samples were dried at room temperature (25ºC) or in an oven at a maximum temperature of 50˚C. In addition, the digestion of the samples was done in a closed system (microwave oven) to avoid losing the mercury concentration.

The seeds of all three plants were analyzed in order to determine mercury concentration, the results being negative. A quantity of 0.5 g of seeds was mineralized with 9 mL nitric acid (HNO_3), 1 mL hydrogen peroxide (H_2O_2) and 1 mL hydrochloric acid (HCl), in a closed system.

The digestion process was applied in a microwave system following an initial digestion program given by the equipment manufacturer (0.5 g plant and 9 mL HNO_3, 1 mL H_2O_2 at 180º C). Because the recovery yields on a matrix-type CRM (BCR-482 Lichen) were extremely low, the extraction method was improved by increasing the digestion temperature by 10 degrees and adding 1 mL of HCl to stabilize the mercury in the liquid system. After optimizing the method, a mixture of 9 mL HNO_3, 1 mL HCl, 1 mL H_2O_2 was used.

The development of a method for the determination of mercury in plants using atomic fluorescence spectrometry (AFS) was pursued. The determination of mercury content from both standards and matrix-type MRCs resulted in recovery yields of 50-60%. It was concluded that the H_2O_2 used in the digestion process interferes in the determination of mercury by the method mentioned above, because the other reagents used in the digestion method (HNO_3 and HCl) are usual reagents from the AFS method. It was assumed that the plant matrix is not suitable for the determination of Hg using AFS technique.

For the extraction and digestion of plant samples, the addition of H_2O_2 is essential in the mineralization process. That is why it was decided to change the mercury determination method, namely through the CV-AAS technique, where the recovery yields on both the matrix-type CRMs and on the standards were very good (103.3 ± 2.0 % for CRM NIST 1515 and 105.6 ± 1.5 % for CRM BCR-482). These improvements were made gradually, so that in the end the optimal digestion method is the one presented in table 4.

The Hg extraction from the plant material was carried out as follows: the plant's organs were dried in a thermostated oven at 50ºC. 9 mL of HNO_3, 1 mL of HCl and 1 mL of H_2O_2 were added to approximately 1.0 g dried plant. The samples were introduced into a closed digestion system (microwave digestion system). After mineralization, the samples were filtered through 0.45 µm filters, the obtained solution being brought to the mark with ultrapure water in a 50 mL volumetric flask.

Tabel 4. Vegetable tissue digestion program for Milestone ETHOS UP microwave system

Step	Time (min)	Temperature (ºC)	Power (W)
1	20	190	1800
2	15	190	1800

Mobile inorganic and organic Hg concentration in soils

To determine the mobile fraction of inorganic mercury, an acidic solution (pH=5) of 1M $CaCl_2$ was used under specific conditions: 5g of the soil sample was weighed, over which 50 mL of 1M $CaCl_2$ solution was added, the samples being shaken on a shaker for 2h at 40 rpm, at a constant temperature of 20º. Two samples were made for each experiment, thus calculating the average between the two determinations.

To determine the mobile fraction of total mercury, an acidic solution of 2% HCl and 10% ethanol was used, which was brought to a pH in the range of 1 to 3 pH units. 1.5g of the soil sample was added to 2.5 mL of the acidic solution. The samples were introduced into a centrifuge for 10 minutes at 3000 rpm, repeating this procedure 3 times. After centrifugation, the samples were heated using a heating process in an ultrasonic thermostat bath at 60º C for 7 minutes. The samples were left to cool, then the supernatant was filtered through a 0.45 µm filter and then brought to the mark with HNO_3 0.5%. Two samples were made for each experiment, thus calculating the average between the two determinations.

The mobile fraction of organic mercury was calculated by the difference between the mobile fraction of total mercury and the mobile fraction of inorganic mercury.

Total content of Hg in soil

The procedure applied for extraction of total Hg from soils was in accordance with in force legislation [21].

Method for Hg determination in plants

In order to estimate the content of Hg (total, inorganic, organic) in plants, a method using cold vapor atomic absorption spectrometry (CV-AAS) was applied. For the method were tested parameters the following parameters: linearity, working range, dispersion homogeneity test; detection limit (LOD); quantification limit (LOQ); repeatability (RSDr); intermediate precision (RSDR); uncertainty measurement (Uex); recovery.

The validated method was used for quantification of Hg from the plant's extracts and CRMs matrices.

RESULTS AND DISCUSSION

Performance parameters for the applied method for Hg determination in plants

A calibration curve was drawn in the range 1 µg/L to 10 µg/L, using Hg standard in 1% HNO_3 solution. In the following paragraph, the internal validation data for the mercury determination method by cold vapor atomic absorption spectrometry (CVAAS) are presented. The value of performance parameters are presented in table 5 and the linear regression curve in figure 2.

Table 5. Performance parameters of the mercury determination method from vegetation samples

Working range, µg/L	LOD, µg/kg	LOQ, µg/kg	RSDr %	RSDR %	Uex %	Recovery,%
1÷10	5	15	5.0	8.0	19.0	103.3 ± 2.0 NIST 1515 105.6 ± 1.5 BCR-482

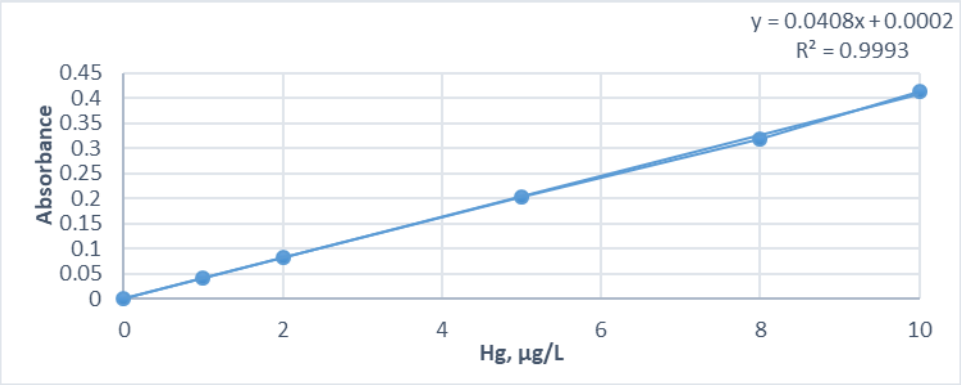

Figure 2. Linear regression curve for Hg using CV-AAS

Content of total and mobile Hg in soils

In table 6 are presented the Hg concentration in blank samples and enriched soil samples used in T1 and T2 experiments on the beginning of the experiments. The results represent average of three different determinations and the uncertainty is reported.

Table 6. Hg content in soil samples (control soils, soils after enrichment and polluted soil) on the beginning of the experiments, mg/kg

Sample	Blank tests*	T1/1m	T1/2m	T2/1m	T2/2m	VC1	VC2
Value	<0.05**	**1.05**±0.20	**1.01**±0.19	**3.87**±0.74	**3.93**±0.75	**0.21**±0.04	**0.23**±0.05

***M1m, M2m, M1t, M2t, M1wt, M2wt;

** Quantification limit of Hg in soil using CV-AAS method.

At the end of the experiments for all plants, the total mercury concentration in the soil was determined, as well as its mobile fraction (organic and inorganic mercury). The same procedure was applied at the end of the experiment as at the beginning to estimate the mercury concentration. The results are presented in table 7.

Table 7. Mobile and total content of Hg at the end of the experiments

Experiments	Total mercury	Organic mercury	Inorganic mercury
Blank test	<0.05*	<0.05*	<0.05*
T1/1m	**0.98**±0.19	<0.05 (**0.018**)	<0.05
T1/2m	**0.92**±0.17	<0.05 (**0.016**)	<0.05
T2/1m	**3.84**±0.73	<0.05 (**0.045**)	<0.05
T2/2m	**3.73**±0.71	**0.052**±0.006	<0.05
VC1m	**0.20**±0.04	<0.05	<0.05
VC2m	**0.21**±0.04	<0.05	<0.05
VC1t	**0.22**±0.04	<0.05	<0.05
VC2t	**0.21**±0.04	<0.05	<0.05
VC1wt	**0.19**±0.04	<0.05	<0.05
VC2wt	**0.23**±0.05	<0.05	<0.05

* Quantification limit of Hg in soil using CV-AAS method

From the results presented in table 7 it can be observed that inorganic mercury is not bioavailable for plants uptake.

The total Hg content in the soil at the end of the experiment (table 7) remains practically unchanged compared to the one at the beginning of the experiment (table 6).

Determination of mercury concentration in mustard experiments

To determine the concentration of mercury in the mustard plants, the samples were taken after 1 month, respectively 3 months of exposure. At each sampling, one plant was taken, then the plant

was washed with ultrapure water (figure 2) and the components (root, stem, leaves) were separated (figure 3). The vegetal material was treated as was presented in *Pre-treatment of soils and plant's organs* section.

a) M1m, M2m **b) T1/1m** **c) T1/2m** **d) T2/1m** **e) T2/2m**

Fig. 2. Sampling of mustard plants (after 1 month of experiment)

Fig. 3. Separation of mustard plants by components (root, stem, leaves)

The following table shows the results obtained after analyzing the vegetation samples.

Table 7. Mercury concentrations obtained in mustard samples, µg/kg

Sample analyzed	Hg concentration after 1 month			Hg concentration after 3 months		
	root	stem	leaves	root	stem	leaves
M1m	<15.0	<15.0	<15.0	<15.0	<15.0	<15.0
M2m	<15.0	<15.0	<15.0	<15.0	<15.0	<15.0
T1/1m	<15.0 (**10**)*	<15.0 (**5**)	**20 ± 3.80**	<15.0 (**10**)	<15.0 (**10**)	<15.0
T1/2m	<15.0 (**10**)	<15.0	<15.0	<15.0 (**10**)	<15.0 (**9**)	<15.0 (**6**)
T2/1m	<15.0	<15.0 (**7**)	<15.0	<15.0 (**8**)	<15.0	<15.0
T2/2m	**20 ± 3.80**	<15.0	<15.0	<15.0	<15.0	<15.0
VC1m	**50 ± 9.50**	<15.0 (**10**)	**40 ± 7.60**	<15.0	<15.0 (**5**)	<15.0
VC2m	**50 ± 9.50**	<15.0 (**10**)	<15.0 (**10**)	<15.0	<15.0	<15.0

* values reported in brackets are informative, being lower than quantification limit

The translocation coefficient from soil to root (TC), the bioaccumulation factors from the root to the leaves and from the root to the stem (TF) were calculated for all the experiments carried out. TC and TF were calculated according to literature data [22]. The values reported were situated in the range of 0.01 to 0.05, thus demonstrating the fact that mercury does not accumulate in any part of mustard plants, regardless of whether the plants were grown in artificially contaminated soil or natural soil with Hg content.

Determination of mercury concentration plants from experiments with thyme and wild thyme

The vegetal material was collected after one month and three months for both species. The procedure for collection the plants was the same used for mustard experiments. All samples of vegetation materials (root, stem, leaves) recorded Hg values lower than detection limit (5 µg/kg), not only lower than quantification limit (15 µg/kg). We can conclud that in case of thyme and wild thyme, mercury did not accumulate in any part of the plant.

CONCLUSIONS

The study proposed a method for determination of mercury from vegetation materials (plants) using cold vapor atomic absorption spectrometry technique. In addition, the paper propose an improved pretreatment method for digestion of plants in order to evaluate Hg content.

The translocation and bioaccumulation experiments were performed using three different types of plants such as, mustard, thyme and wild thyme. The results indicated that none of these plants bioaccumulated mercury, the bioaccumulation factors from the root to the leaves and to the stem had values approximately equal to 0.

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